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ORIGINAL ARTICLE

Molecular design of phosphatidylserine-inspired polymers for efficient anti-inflammatory therapy via enhanced interaction with Tim-4

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Abstract

This study investigated the interaction between phosphatidylserine (PS)-inspired polymers, T-cell immunoglobulin and mucin-like domain-containing protein 4 (Tim-4) by systematically varying the monomer structure and copolymer composition. A series of alkyl-substituted PS-inspired monomers was synthesized using a modified phosphoramidite method, and well-defined homopolymers and 2-hydroxyethyl methacrylate (HEMA)-containing copolymers were prepared via reversible addition–fragmentation chain-transfer polymerization. Structural analyses using ^{1}H nuclear magnetic resonance and gel permeation chromatography confirmed the successful synthesis with controlled molecular weights. Biolayer interferometry was used to quantify Tim-4 binding, revealing a nonmonotonic effect of alkyl substitution, whereas the incorporation of HEMA consistently enhanced Tim-4 binding in a composition-dependent manner. Biological evaluation using RAW-Blue macrophages revealed that the homopolymers did not significantly affect interleukin-6 (IL-6) secretion, whereas the copolymers selectively suppressed IL-6 production. Notably, the copolymer containing 50 mol% PS units exhibited the strongest IL-6 suppression, and the HEMA-containing copolymers exhibited anti-inflammatory activity even at lower PS concentrations than the homopolymers did. These results demonstrate that the copolymer composition critically influences receptor interactions and immune modulation. This study highlights the potential of PS-inspired copolymers as biomaterials that mimic apoptotic cell signals and exert efficient anti-inflammatory effects through an optimized molecular design.

Introduction

Apoptotic cells are continuously generated in the body and are cleared by phagocytes via efferocytosis [1]. During this process, inflammation is not only prevented but also actively suppressed by the induction of anti-inflammatory cytokines such as transforming growth factor β and interleukin-10 [2, 3]. Unlike steroids or nonsteroidal anti-inflammatory drugs, the phagocyte-dependent resolution of inflammation has attracted attention as a novel therapeutic approach [4, 5]. A hallmark of apoptotic cells is the "eat-me" signal of exposed phosphatidylserine (PS) on the outer leaflet of the plasma membrane [6]. PS is recognized by T-cell immunoglobulin domain and mucin-like domain (TIM) family receptors, TAM receptor tyrosine kinases (MerTK, Axl, and Tyro3), and integrin complexes [7]. Among these, T-cell immunoglobulin and mucin-like domain-containing protein 4 (Tim-4) binds PS with high affinity but lacks a cytoplasmic signaling domain; it functions primarily as a tethering receptor that cooperates with the MerTK and integrin pathways to drive engulfment [8–10]. Structural studies have revealed that the Tim-4 immunoglobulin variable (IgV)-like domain contains a metal ion-dependent ligand-binding site (MILIBS) and that

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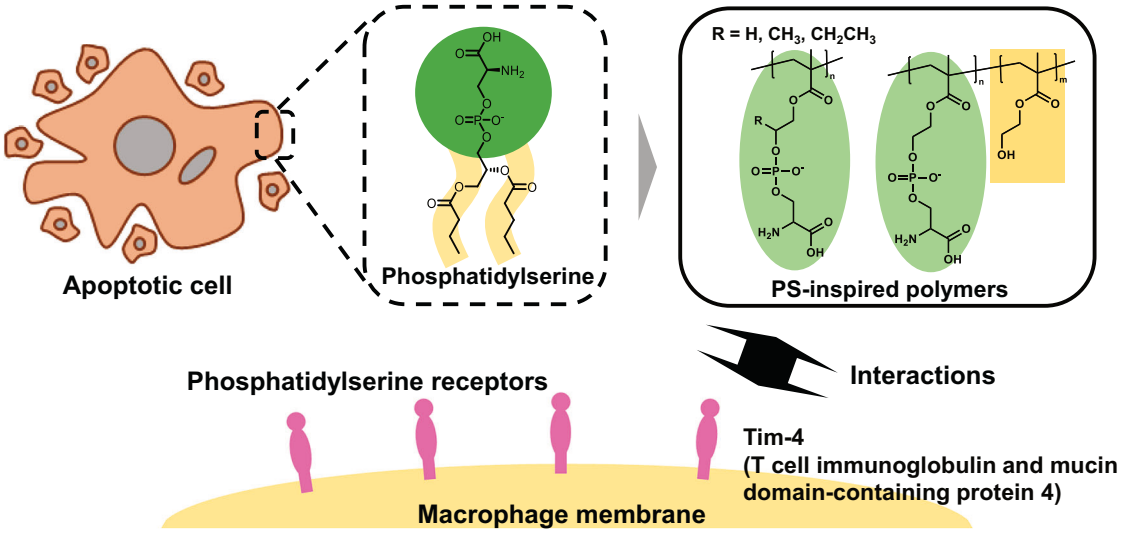


Fig. 1 Conceptual diagram of the study, demonstrating the molecular interactions between Tim-4 and PS-inspired polymers, focusing on how the polymer structure influences receptor binding and downstream anti-inflammatory response

hydrophobic interactions with PS acyl chains stabilize membrane engagement [11, 12]. These findings highlight how the chemical architecture of PS governs its receptor binding.

PS-inspired polymers have been developed on the basis of apoptotic cell membranes. Polymers bearing 2-methacryloyloxyethyl phosphorylserine (MPS) mimic the phosphorylated serine headgroups and suppress the inflammatory activation of macrophages and microglia as polymers or particles [13–15]. Copolymer design is also known to improve biocompatibility and function by tuning hydrophilicity and conformation, reducing nonspecific protein adsorption, and optimizing ligand presentation [16]. For example, adjusting the ligand density and spacing in glycopolymers dramatically modulates lectin recognition [17], a multivalency and optimal-density concept likely transferable to PS-inspired systems for receptor recognition and anti-inflammatory efficacy.

The monomer structure can strongly affect receptor binding [18]. Although the Tim-4 IgV-like domain recognizes PS within a defined pocket [11], variations in the fatty acyl chain length or hydrophobic substitutions modulate its affinity for PS-containing membranes [19, 20]. Thus, alkyl substitution on MPS monomers could alter the PS-like display and hydration, thus affecting receptor engagement and affinity. Moreover, the copolymerization of MPS with hydrophilic comonomers (e.g., 2-methacryloyloxyethyl phosphorylcholine) can enhance anti-inflammatory outcomes relative to those of homopolymers, presumably by better matching the charge and hydration environment of apoptotic membranes and strengthening PS–receptor interactions [21, 22]. Taken together, these observations indicate that both the alkyl architecture of MPS monomers and the copolymer composition play important roles in modulating PS–receptor interactions and the resulting immunomodulatory functions.

On the basis of these results, we hypothesized that both the monomer-level structure (alkyl substitution) and the copolymer-level design (composition) would jointly regulate Tim-4 binding and the anti-inflammatory functions of PS-inspired polymers (Fig. 1). Therefore, we synthesized alkyl-substituted PS-inspired monomers, well-defined homopolymers, and 2-hydroxyethyl methacrylate (HEMA) copolymers via reversible addition–fragmentation chain-transfer (RAFT) polymerization. These polymers were subsequently evaluated for their Tim-4 binding and immunomodulatory activity to determine their structure–function relationships.

Materials and methods

Materials

O-tert-butyl-*N,N,N',N'*-tetraisopropyl phosphorodiamidite and 4-cyano-4-(((dodecylthio)carbonothioyl)thio)pentanoic acid (CDSPA) were purchased from Sigma–Aldrich (USA). *N,N*-Dimethylformamide (DMF), HEMA, 2-hydroxypropyl methacrylate (HPMA), dichloromethane (DCM), imidazole hydrochloride, and 2,2′-azobis(isobutyronitrile) (AIBN) were obtained from FUJIFILM Wako Pure Chemical (Osaka, Japan). 2-Hydroxybutyl methacrylate (HBMA) and trifluoroacetic acid (TFA) were purchased from Tokyo Chemical Industry (Tokyo, Japan). *N*-Boc-*L*-serine *tert*-butyl ester was obtained from ChemImpex International (USA). Recombinant mouse Tim-4/human Fc chimera was

purchased from FUJIFILM Wako. Lipopolysaccharide (LPS; *E. coli* K12, ultrapure), QUANTI-Blue, an interleukin-6 (IL-6) enzyme-linked immunosorbent assay (ELISA) kit, and RAW-Blue cells were obtained from Invitrogen (USA). An MTT assay kit was purchased from Nacalai Tesque (Kyoto, Japan). HEMA, HPMA, and HBMA were distilled to remove inhibitors and then stored under nitrogen at 4 °C until use. The water was subsequently purified (Milli-Q). Dulbecco's phosphate-buffered saline (D-PBS; Nacalai Tesque) was used to prepare the buffers.

Synthesis of PS-inspired monomers

PS-inspired monomers were synthesized using a modified phosphoramidite method [21]. In DCM (129 mL), imidazole hydrochloride (0.574 g, 5.49 mmol), *O-tert*-butyl-*N,N,N',N'*-tetraisopropyl phosphorodiamidite (5.243 g, 17.22 mmol), and *N*-Boc-*L*-serine *tert*-butyl ester (5.00 g, 24.36 mmol) were stirred for 21 h at room temperature under nitrogen. HEMA (2.205 mL, 18.18 mmol), HPMA (2.551 mL, 18.18 mmol), or HBMA (2.824 mL, 18.18 mmol), along with imidazole hydrochloride (1.88 g, 17.98 mmol), were added and allowed to react for 150 min, after which the same amount of imidazole hydrochloride was added at 45 and 90 min. After quenching, the mixture was washed with brine, and the organic phase was dried over anhydrous Na_2SO_4 overnight, concentrated, purified using silica gel chromatography, and characterized using ^1H nuclear magnetic resonance (NMR; JEOL, 400 MHz).

Synthesis of PS-inspired polymers

The homopolymers and HEMA copolymers were prepared via RAFT polymerization. Monomer feeds (PS-inspired monomer alone, or eMPS:HEMA = 100:0, 75:25, 50:50, 1:99), CDSPA, and AIBN were dissolved in DMF:EtOH = 1:1 (v/v) at a total monomer concentration of 0.5 M, with [CTA]:[AIBN] = 5:1. After nitrogen purification for 30 min, the polymerization proceeded at 60 °C for 20 h. Crude products were purified by dialysis (MWCO 3.5 kDa): first against DCM for 48 h, followed by deprotection with TFA (25 v/v% of dialysate) at 25 °C for 8 h to remove the *tert*-butyl and Boc groups. The solvents were evaporated, and the residue was dialyzed against 0.1 M NaOH for 24 h and Milli-Q water for 24 h and then lyophilized. Polymerization and deprotection were verified by ^1H NMR spectroscopy and gel permeation chromatography (GPC; TSK-GEL; polystyrene standards Mw 500–2,110,000).

Tim-4 binding evaluation by biolayer interferometry

The interactions between Tim-4 and the polymers were quantified by biolayer interferometry (BLI) using an Octet R2 system (ForteBio) with APS biosensors. Sensors were equilibrated in D-PBS, and mouse Tim-4/human Fc chimera (FUJIFILM Wako, 1 mg/mL in D-PBS) was immobilized on the sensors for 20 min. After a brief rinse in D-PBS, association was measured by dipping the sensors into polymer solutions (in D-PBS) for 20 min, followed by dissociation in D-PBS for 10 min at 25 °C with shaking at 1000 rpm. Reference subtraction was performed using matched reference sensors and buffers. Kinetic data were fitted using a 1:1 Langmuir global binding model to obtain the dissociation constant (K_D), and steady-state analysis was applied to the weak binders.

Cytotoxicity (MTT) assay

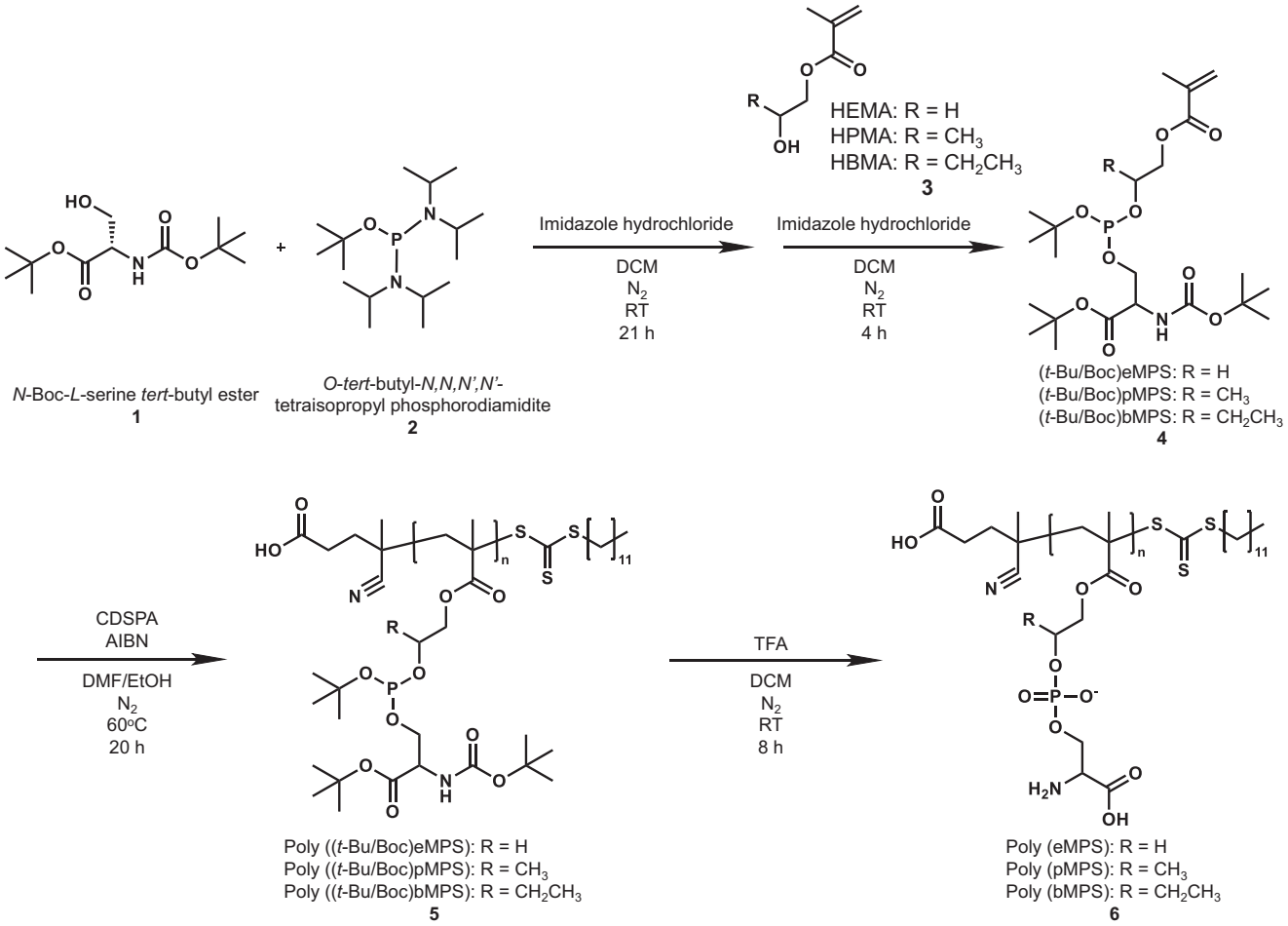
RAW-Blue cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C in 5% CO_2. Cells (5×10^4 cells per well) were seeded into 96-well plates. After 24 h, the wells were washed with D-PBS, and 150 µL of fresh medium containing 30 µL of the polymer sample and IL-4 (positive control) was added. Following an additional incubation period of 24 h, the medium was aspirated, and 10 µL of the MTT solution and 100 µL of the medium were added over the course of 2 h. Subsequently, 100 µL of solubilization solution was added, and the absorbance was measured at 570 nm with a 670 nm reference wavelength.

Anti-inflammatory assays (NF-κB and IL-6)

RAW-Blue cells (5×10^4 per well) were seeded in 96-well plates and incubated for 24 h. The cells were stimulated with LPS (final concentration: 0.24 µg mL^{-1}; 10 µL of LPS in 4 µg mL^{-1} D-PBS per well) for 30 min and then washed once with D-PBS, after which 150 µL of fresh medium was added. Polymer samples and IL-4 (30 µL) were added, and the supernatants were collected after 24 h. IL-4 was used as a positive control because of its known ability to induce anti-inflammatory responses. NF-κB activity was quantified using the QUANTI-Blue assay: 20 µL of the supernatant was mixed with 180 µL of QUANTI-Blue solution and incubated at 37 °C for 60 min, after which the absorbance was measured at 620 nm. IL-6 levels were measured using ELISA according to the manufacturer's instructions, and the absorbance was read at 450 nm (reference wavelength: 570 nm).

Statistical analysis

Unless otherwise noted, the data are presented as the means ± standard deviations. Multiple comparisons were performed using Tukey's test implemented in EZR, a graphical interface for R developed at the Jichi Medical



Scheme 1 Synthesis of alkyl-substituted phosphorylserine monomers (eMPS, pMPS, bMPS) via a modified phosphoramidite method, followed by RAFT polymerization to produce well-defined alkyl-PS homopolymers with controlled molecular weights

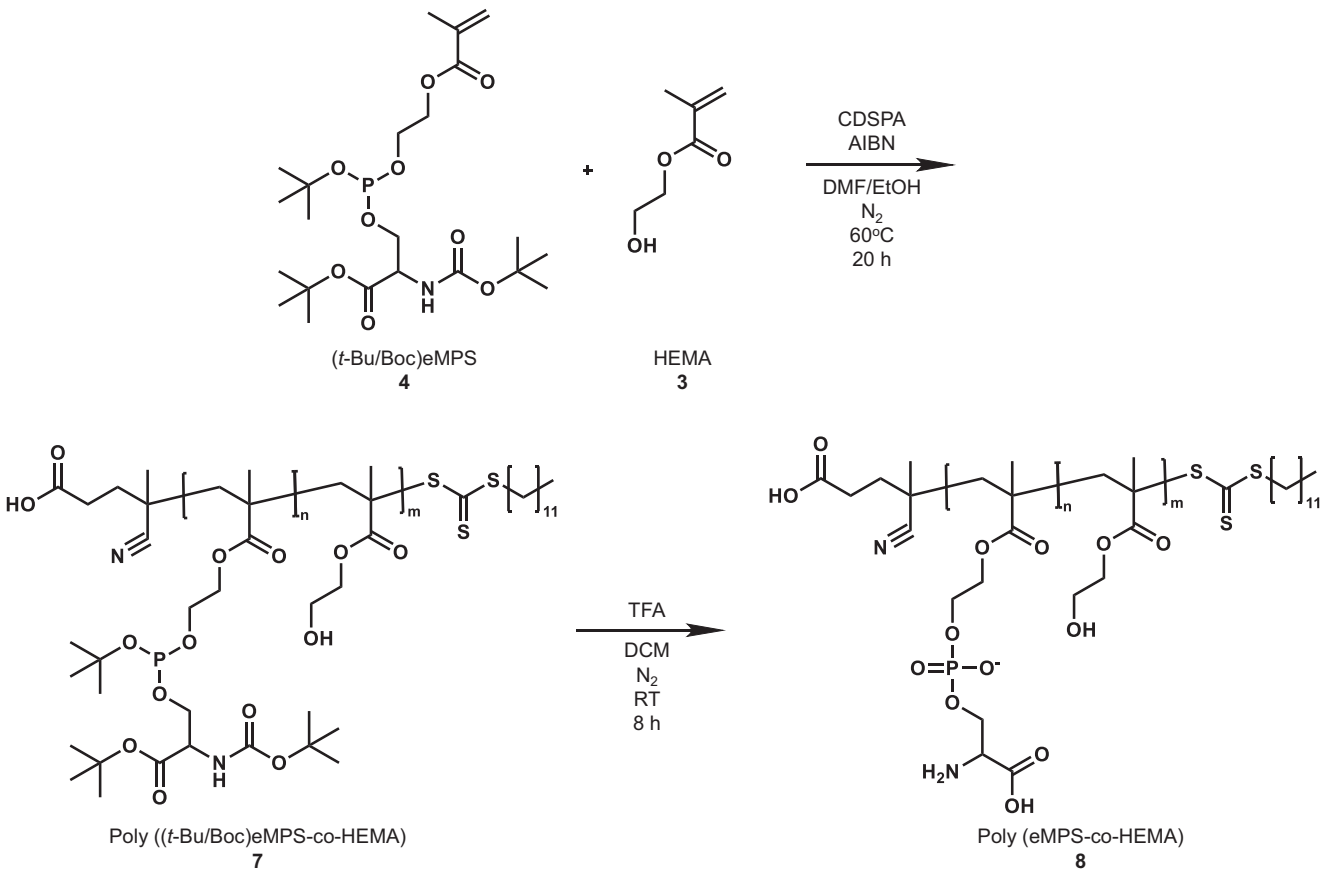
University Saitama Medical Center. EZR is a modified version of R Commander and is designed to incorporate statistical functions commonly used in biostatistics. Statistical significance was set at $p < 0.05$ [23].

Results and discussion

Synthesis and characterization of PS-inspired polymers

The incorporation of PS-like functional groups to mimic apoptotic membranes has previously been shown to attenuate inflammatory responses in macrophages and microglia [13, 14]. Building on this approach, we introduced phosphorylserine moieties into methacrylate scaffolds using phosphoramidite chemistry to synthesize alkyl-controlled PS-inspired monomers [21]. Specifically, 2-methacryloyloxyethyl phosphorylserine (eMPS), 2-methacryloyloxypropyl 2-phosphorylserine (pMPS), and 2-methacryloyloxybutyl 2-phosphorylserine (bMPS) were prepared by varying the alkyl-derived comonomers HEMA, HPMA, and HBMA. RAFT polymerization was then used to produce the corresponding alkyl-PS homopolymers (Scheme 1). Additionally, the copolymerization of eMPS with HEMA yielded composition-controlled com-PS copolymers (Scheme 2). HEMA was selected for its ability to maintain hydrophilicity and has been previously incorporated into p(HEMA-*co*-MPS) antibody conjugates without compromising its anti-inflammatory activity [22].

In the ^{1}H NMR spectra of the alkyl-PS monomers, methyl/propyl-derived resonances confirmed the structures, and integration verified the identities of the monomers (Fig. S1). For the com-PS copolymers, the compositions were calculated from the integrals of HEMA hydroxyl-derived protons and backbone/protecting-group signals. GPC showed Mn = 5800–9600 g mol^{-1} and polydispersity indices (PDIs) of 1.1–1.4 for alkyl-PS (Table 1) and Mn = 4700–10,300 g mol^{-1} and PDI = 1.3–1.5 for the copolymers (Table 2).



Scheme 2 RAFT copolymerization of eMPS with 2-hydroxyethyl methacrylate (HEMA) to yield composition-tunable com-PS copolymers

Table 1 GPC-derived molecular weights and PDIs for alkyl-PS homopolymers

	M_n (g mol^{-1})[a]	M_w (g mol^{-1})[a]	M_w/M_n[a]
Poly ((*t*-Bu/Boc)eMPS)	5.8×10^3	6.8×10^3	1.1
Poly ((*t*-Bu/Boc)pMPS)	9.6×10^3	13.0×10^3	1.4
Poly ((*t*-Bu/Boc)bMPS)	8.4×10^3	9.0×10^3	1.1

[a]Determined by GPC using DMF in 10 mM lithium bromide (LiBr) and calculated using a polystyrene standard

Table 2 GPC-derived Mn and PDI and 1H NMR spectroscopy-derived compositions of com-PS copolymers

In feed (mol%)[a]				
MPS	HEMA	M_n (g mol^{-1})[b]	M_w (g mol^{-1})[b]	M_w/M_n[b]
77	23	4.7×10^3	6.4×10^3	1.3
66	34	8.8×10^3	12.6×10^3	1.4
50	50	10.3×10^3	15.0×10^3	1.5
3	97	8.6×10^3	11.1×10^3	1.3

[a]Calculated using 1H NMR spectroscopy, Solvent: DMSO-d6, Conc.: 10 mg/mL, 400 MHz

[b]Determined by GPC using DMF in 10 mM lithium bromide (LiBr), calculated using a polystyrene standard

Tim-4 binding affinity

Tim-4 recognizes PS via MILIBS within its IgV-like domain [11] and functions primarily as an accessory/tethering receptor [9]; these structure–function features are well established [8, 11]. BLI revealed that the binding response of the alkyl-PS polymers increased in the order of pMPS < eMPS < bMPS, indicating a nonmonotonic trend with respect to simple alkyl substitution (Fig. 2a). This behavior indicates that Tim-4 is not governed solely by the presence of PS but is strongly dependent on local PS density, presentation mode, and physical properties. The binding of TIM family members depends on local membrane properties, including the insertion of a hydrophobic loop [19]. Furthermore, the response of Tim-4 varies significantly depending on the PS density and lipid phase, and these factors have been demonstrated to systematically govern binding dynamics [20]. These findings indicate that differences in self-association or aggregation in aqueous solutions alter the density of the accessible PS-like epitopes, thus producing the observed nonmonotonic trend. In

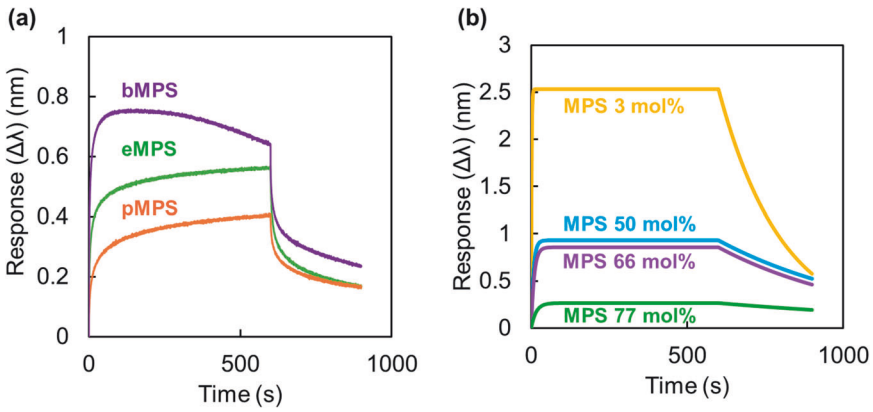


Fig. 2 Bindings of polymers to Tim-4 evaluated by BLI. **a** Sensorgrams (association/dissociation) for alkyl-PS homopolymers. **b** Increased responses with higher HEMA fractions in com-PS copolymers. Kinetic fits were performed using a 1:1 Langmuir model. Each copolymer corresponds to the following composition ratios (MPS mol%:HEMA mol%): (3:97), (50:50), (66:34), and (77:23)

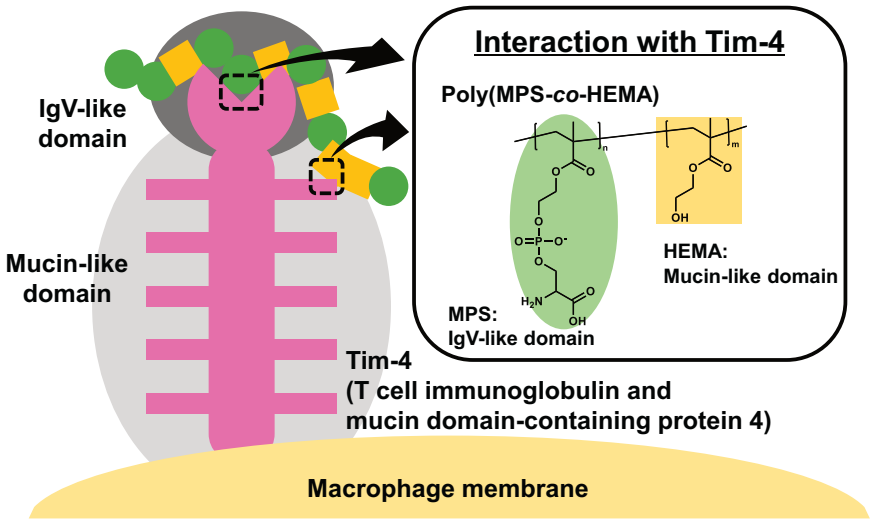


Fig. 3 Conceptual schematic illustrating the proposed interactions between HEMA segments in com-PS polymers and the mucin-like domain of Tim-4. The diagram highlights how HEMA incorporation into PS-inspired copolymers may enhance receptor binding by stabilizing interactions beyond primary PS recognition

addition, the geometric and curvature cues of apoptotic membranes modulate efferocytic receptor recruitment efficiency [19], supporting an optimal density/arrangement hypothesis applicable to this polymer system.

In contrast, Tim-4 binding increased with increasing HEMA content in the com-PS polymers (Fig. 2b). This may reflect secondary interactions between HEMA and the mucin-like domain of Tim-4 [24] and is in accordance with the multivalency/optimal-density principles widely observed in glycan–lectin systems [17] (Fig. 3). A more hydrated HEMA-rich matrix likely mitigates excessive hydrophobic association or aggregation among PS-like groups, thus enhancing the effective epitope valency of Tim-4 [25]. Nevertheless, because Tim-4 cooperates with integrins and TAM receptors during engulfment, enhanced Tim-4 binding alone is unlikely to be sufficient to increase functional PS signaling [10].

Cytotoxicity toward macrophages

In RAW-Blue cells, alkyl-PS polymers exhibited no detectable toxicity at concentrations ≤5 mM (PS-unit basis), but cell viability decreased at concentrations ≥10 mM (Fig. 4). Similarly, the com-PS copolymers were nontoxic at concentrations up to 5 mM (Fig. S2). These results are consistent with those of previous studies on phosphorylserine-inspired polymers and phospholipid-based materials, which generally demonstrated good biocompatibility at low to moderate concentrations but may induce cytotoxic effects at higher doses [13, 14]. For instance, polymers bearing MPS have shown minimal cytotoxicity in macrophages and microglia below 5 mM [13, 14].

The lack of toxicity at functional concentrations (≤5 mM) supports the potential of these PS-inspired polymers for biomedical applications, where minimizing adverse effects on immune cells is critical. Furthermore, the incorporation of hydrophilic comonomers such as HEMA may enhance biocompatibility by improving polymer solubility and reducing nonspecific interactions with cell membranes, as observed in other copolymer systems [16, 25].

Anti-inflammatory effects: NF-κB and IL-6

The alkyl-PS homopolymers did not significantly suppress IL-6 at 5 mM (Fig. 5a), indicating limited anti-inflammatory

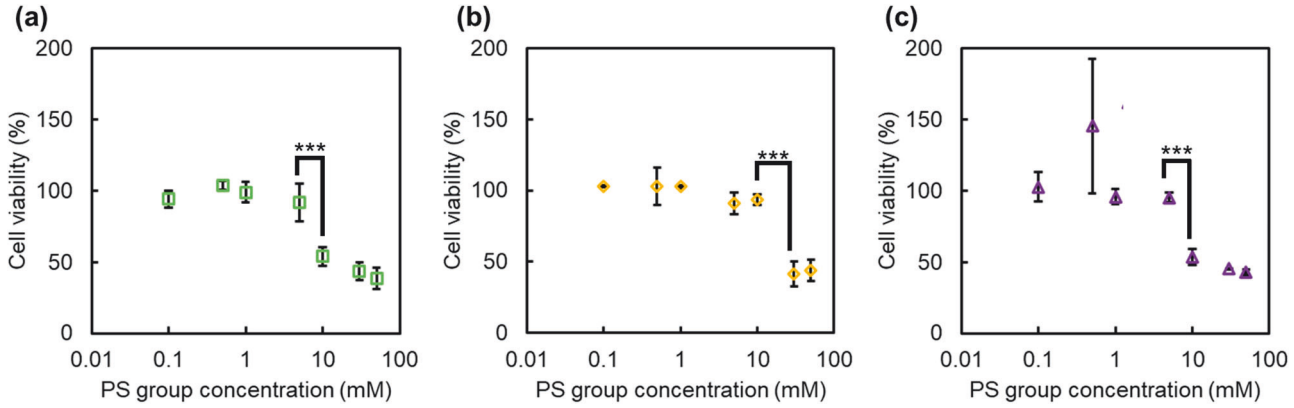


Fig. 4 Evaluation of the cytotoxicity of alkyl-PS homopolymers toward RAW-Blue macrophages as a function of PS unit concentration (0.1–50 mM). After 24 h of incubation, cell viability was assessed by an MTT assay. **a** eMPS, **b** pMPS, and **c** bMPS

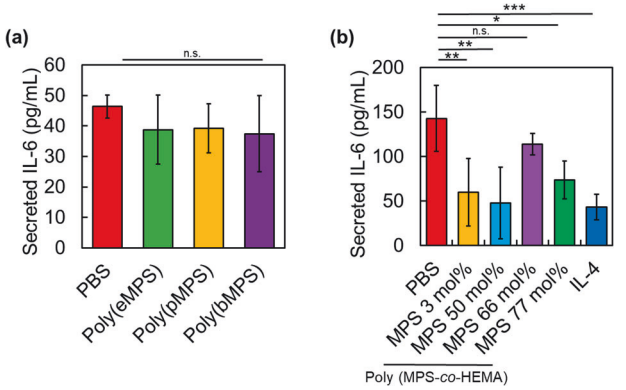


Fig. 5 Secreted IL-6 levels normalized to cell viability in RAW-Blue macrophages. **a** Alkyl-PS homopolymers at a PS concentration of 5 mM did not significantly suppress IL-6 secretion. **b** Com-PS copolymers at a unit concentration of 3.3 mM selectively suppressed IL-6 secretion, with maximal inhibition observed at 50 mol% PS content. Each copolymer corresponds to the following composition ratios (MPS mol%:HEMA mol%): (3:97), (50:50), (66:34), and (77:23)

activity at this concentration. In contrast, the com-PS copolymers exhibited a selective immunomodulatory profile: while NF-κB activity remained largely unchanged (Fig. S3), IL-6 production was significantly suppressed at the same dose, with the most pronounced effect observed in copolymers containing 50 mol% PS units (Fig. 5b).

The stable NF-κB levels and reduced IL-6 secretion indicate pathway specificity rather than broad suppression of inflammatory signaling. IL-6 is a canonical NF-κB target gene; however, its expression is also tightly regulated by the IL-6/STAT3 positive feedback loop, which can modulate IL-6 activity independently of upstream NF-κB activation [26]. The enhanced hydration and spatial presentation of PS units in the copolymers plausibly influence receptor clustering or endocytic trafficking, thus indirectly modulating STAT3 signaling pathways. This mechanism aligns with previous reports of the polymer architecture influencing downstream signaling cascades beyond initial receptor binding [17].

Furthermore, similar anti-inflammatory properties of PS-inspired polymers have been reported in microglia, supporting the notion that these materials induce cell-type-independent immunomodulation [14]. These polymers mimic the "eat-me" signal of apoptotic cells, which is known to actively promote the resolution of inflammation via multiple receptor-mediated pathways, including those involving TAM receptors and integrins, in addition to TIM family members [1, 8].

Previous studies have also highlighted that the anti-inflammatory efficacy of PS-mimetic materials depends on their ability to modulate not only receptor affinity but also downstream signaling dynamics, reinforcing the importance of precise copolymer composition and molecular design [21, 22].

Correlating Tim-4 binding affinity with anti-inflammatory activity

No direct correlation was observed between Tim-4 binding affinity and the degree of IL-6 suppression, as measured by the BLI response (Fig. 6a). These findings align with those of previous studies: although Tim-4 engagement is necessary for apoptotic cell recognition, it is insufficient for driving downstream anti-inflammatory signaling, given the critical involvement of coreceptors such as MerTK and integrin complexes in efferocytosis [10]. Multicomponent receptor interplay likely introduces complexity beyond simple binding-affinity metrics.

When IL-6 suppression was analyzed as a function of the PS unit concentration, the copolymers consistently outperformed the homopolymers, achieving significant anti-inflammatory effects even at lower PS densities (Fig. 6b). This enhanced efficacy can be attributed to the multivalency

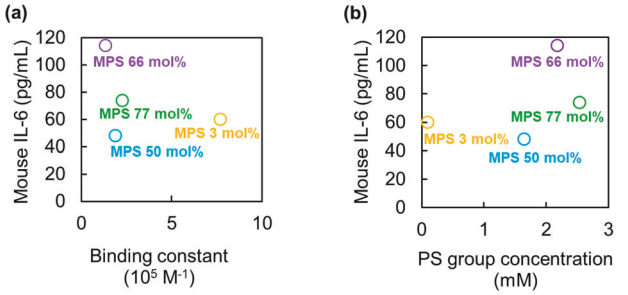


Fig. 6 Relationships between binding and function. **a** Lack of direct correlation between the BLI response and IL-6 suppression. **b** IL-6 suppression vs. PS group concentration, showing the superior potency of com-PS, even at low PS contents. Each copolymer corresponds to the following composition ratios (MPS mol%:HEMA mol%): (3:97), (50:50), (66:34), and (77:23)

and optimized spatial presentation of the PS moieties within the hydrophilic copolymer matrix, which likely promotes more effective receptor clustering and engagement. Such multivalent interactions are well known to increase receptor-binding avidity and signaling potency in glycopolymer and ligand–receptor systems [17, 25]. In addition, HEMA, incorporated as a comonomer in this study, has been reported to interact with the mucin-like domain of Tim-4 [11], and this interaction likely contributes to the preservation of the anti-inflammatory activity of MPS within the copolymers. However, previous studies have shown that the binding affinity between PS and Tim-4 decreases when the PS content on the liposome surface falls below 10% [20], indicating that a minimum PS density is required for stable receptor interaction. These results indicate that this functional threshold may exist at ~50 mol% PS in the copolymers.

Overall, these results underscore that rational copolymer design not only modulates receptor affinity but also critically influences functional outcome by tuning the ligand density and microenvironmental context. This insight is pivotal for the development of biomimetic materials that faithfully recapitulate apoptotic cell signaling for immunomodulation.

Conclusions

PS-inspired polymers were successfully designed and synthesized by introducing a PS-based functionality via a modified phosphoramidite route. Both alkyl-substituted homopolymers and composition-tunable HEMA copolymers were prepared via RAFT polymerization, and structural characterization confirmed well-defined polymer architectures. Tim-4 binding studies revealed that receptor interactions are modulated in a nonmonotonic manner by alkyl substitution, highlighting the influence of hydrophobicity and polymer assembly. In contrast, HEMA incorporation consistently enhanced Tim-4 affinity in a composition-dependent manner, likely owing to improved hydration and optimized epitope presentation. Biological evaluation revealed that IL-6 secretion was selectively suppressed by com-PS copolymers, with maximal suppression observed at 50 mol% PS. Notably, these copolymers achieved anti-inflammatory effects even at lower PS unit concentrations than the homopolymers did, underscoring the functional advantages of the copolymer design.

Collectively, these findings demonstrate that immunomodulatory activity is governed by both the monomer structure and the copolymer composition. This study advances PS-inspired copolymer design as a promising strategy to mimic apoptotic cell signaling and develop effective anti-inflammatory biomaterials.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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